American Journal of Pathology, Vol. 164, No. 6, June 2004

Plasmodium falciparum-Infected Erythrocytes Adhere Both in the Intervillous Space and on the Villous Surface of Human Placenta by Binding to the Low-Sulfated Chondroitin Sulfate Proteoglycan Receptor

Arivalagan Muthusamy,* Rajeshwara N. Achur,* Veer P. Bhavanandan,* Genevieve G. Fouda,† Diane W. Taylor,† and D. Channe Gowda*

From the Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; and the Department of Biology,† Georgetown University, Washington, DC*

In pregnant women infected with *Plasmodium falciparum*, the parasite-infected red blood cells (IRBCs) sequester in the placenta through chondroitin 4-sulfate (C4S)-mediated adherence. The pattern of IRBC adherence in *P. falciparum*-infected placenta has been controversial. Moreover, the identity of the chondroitin sulfate proteoglycan (CSPG) receptor, that mediates IRBC adherence, and its location in the placenta have not been established. This study, using immunohistochemical techniques, clearly shows, for the first time, that the low-sulfated CSPGs of the placenta are localized predominantly in the intervillous space. *Ex vivo* IRBC adherence analyses demonstrate that the IRBCs are adhered to the CSPG receptors in the placenta in a C4S-dependent manner. This IRBC binding pattern was similar to that observed in *P. falciparum*-infected placentas. These data and the results of dual-fluorescence staining of the endogenous RBCs and syncytiotrophoblasts, and co-localization of CSPG and IRBC adherence unequivocally establish that the low-sulfated CSPGs are the major natural receptors for IRBC adherence in the placenta. Further, it was found that IRBCs adhere mainly in the intervillous space and also at significant levels to the syncytiotrophoblasts. Finally, the *ex vivo* IRBC adherence method described herein provides a reliable procedure for future studies for the assessment of the efficacy of C4S inhibitors and adhesion inhibitory antibodies. *(Am J Pathol 2004, 164:2013–2025)*

Of the four species of *Plasmodium* that infect humans, *P. falciparum* causes the most fatalities.[1–4] This has been attributed to the ability of the infected red blood cells (IRBCs) to sequester in blood capillaries of vital organs, causing severe malaria.[4–8] Various host cell adhesion molecules, including CD36, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, platelet endothelial cell adhesion molecule-1/CD31, thrombospondin on vascular endothelial cell surface, as well as heparan sulfate and chondroitin 4-sulfate (C4S) have been shown to be the receptors for IRBC adherence.[4–15] However, people living in malaria endemic areas acquire, during their childhood, a broad spectrum of protective immunity against malaria, including antibodies that inhibit IRBC adhesion to various receptors.[4,16,17] Therefore, in adults, IRBCs cannot efficiently adhere in the vascular capillaries. To overcome the defensive mechanism of the host, the parasite constantly switches phenotypes by expressing different receptor specificities.[4–8,15,18–20] In the case of pregnant women, *P. falciparum* of a different adherent type selectively adheres to the placenta, causing placental malaria.[21–26] Primigravidas are highly susceptible to placental malaria and the susceptibility decreases with increasing gravidity because of the acquisition of placental malaria-specific immunity during subsequent pregnancies.[26–33]

Although C4S has been shown to mediate IRBC adhesion in the placenta,[34–39] evidence for the chondroitin sulfate proteoglycan (CSPG) receptor type involved in IRBC adherence is lacking. It is well known that, in *P. falciparum*-infected pregnant women, IRBCs sequester in the placenta,[26] and in highly infected placentas, most of the intervillous space is filled with IRBCs. However, the pattern of IRBC adherence is still controversial. Although some investigators have observed IRBC binding to the villous surface, others have found that most IRBCs are localized in the intervillous space and not attached to the syncytiotrophoblasts.[22,26,40,41] *In vitro* studies using snap-frozen placental tissues showed IRBC binding only on the syncytiotrophoblasts.[26,42] This could be because

Supported by the Public Health Service (National Institute of Allergy and Infectious Diseases grants AI45086 to D.C.G. and AI43888 to D.W.T.; and the Fogarty International Center, National Institutes of Health, training grant 5D43TWO1264 to D.W.T.).

A.M. and R.N.A. contributed equally to this work.

Accepted for publication February 6, 2004.

Address reprint requests to D. Channe Gowda, Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033. E-mail: gowda@psu.edu.

of the loss of the intervillous space material during the tissue processing and assay procedures, as suggested previously.[26,42] The presence of fibrous filamentous materials and fibrinoid deposits in the intervillous space of the placental histosections has been reported previously,[26,40] but the possibility that the CSPG receptor present in association with the matrix-like material has not been investigated. It has been proposed that IRBCs present in the intervillous space of *P. falciparum*-infected placentas represent those dislodged from the placental villous surfaces during labor contractions and delivery[26] or IRBCs bound to the C4S chains extending from the syncytiotrophoblasts to the intervillous space.[43] However, considering the surface area of the syncytiotrophoblasts compared to the volume of intervillous space, binding to syncytiotrophoblasts alone cannot explain massive accumulation of IRBCs in the infected placentas. Moreover, because it is widely believed that intervillous space is simply filled with maternal blood without any matrix-like compounds, there is doubt regarding the presence of immobilized CSPGs in the intervillous space. Therefore, there was no satisfactory explanation for the intervillous space IRBC adherence in the infected placentas. Because of these uncertainties, the identity and location of the major natural receptor for IRBC adherence in the placenta remain unresolved.

Human placenta contains three types of CSPGs, high levels of unusually low-sulfated extracellular CSPGs, minor amounts of cell-associated CSPGs, and major amounts of an extracellular dermatan sulfate/chondroitin sulfate proteoglycan (DS/CSPG).[44] Of these proteoglycans, the low-sulfated CSPGs bind IRBCs most efficiently, and therefore, these CSPGs were proposed as the natural receptors.[44] However, there was no direct evidence for the type and location of CSPGs that mediate the IRBC adherence in the placenta. Therefore, this study was undertaken to resolve the above-mentioned controversies and to provide experimental proof that low-sulfated CSPGs are present in the intervillous space and serve as major receptors for IRBC adherence. Immunohistochemical analysis and *ex vivo* IRBC adherence studies using a modified procedure showed, for the first time, that the low-sulfated CSPGs are localized in the intervillous space, and that these are the major natural receptors for IRBC adherence in the placenta. Further, the results of dual-fluorescence staining of the endogenous RBCs and syncytiotrophoblasts, and co-localization of CSPG and IRBC adherence firmly establish that the IRBCs, by binding to the low-sulfated CSPGs, sequester predominantly in the intervillous space and at low but significant levels on the syncytiotrophoblast surface. Additionally, the *ex vivo* adherence assay developed in this study overcomes the problems associated with the preservation of the intervillous space materials and loss of bound IRBCs from the tissue section before examination under the microscope. Thus, the assay procedure is useful for studies testing the efficacy of potential inhibitors of IRBC adhesion and IRBC-adhesion inhibitory antibodies.

Materials and Methods

Tissues and Blood Samples

The blood and placenta tissue samples were collected from the term placentas of *P. falciparum*-infected Cameroonian women, who were admitted for delivery at the Central Hospital, Yaounde, Cameroon. The nature of the project was explained and informed consent was obtained before the collection of the samples. The collection of samples was approved by the Ethical Committee, Ministry of Health, Cameroon, and by the institutional review board at Georgetown University, Washington, DC. Blood samples were cryopreserved in glycerolyte solution, transported, and stored until used. Placental tissues (1×1 cm) were fixed with 10% neutral buffered formalin in phosphate-buffered saline (PBS), pH 7.2. Tissues were cut into 5-μm-thick sections, mounted on glass slides, stained with hematoxylin and eosin (H&E), and examined under light microscope. Normal term placenta tissues were collected, under the Pennsylvania State University College of Medicine IRB approved protocol, from the healthy women who delivered babies at the Hershey Medical Center, Hershey, PA.

Reagents

Protease-free *Proteus vulgaris* chondroitinase ABC (120 U/mg), anti-Δdi-4S mouse monoclonal IgG, and anti-Δdi-6S mouse monoclonal IgM were purchased from Seikagaku America, Falmouth, MA. Anti-Δdi-4S and anti-Δdi-6S antibodies specifically recognize the unsaturated chondroitin sulfate disaccharide stubs, Δdi-4S and Δdi-6S, that formed at C4S and C6S chain attachment regions on core proteins when the proteoglycans were treated with chondroitinase ABC. Bovine tracheal chondroitin sulfate A (52% 4-sulfate, 39% 6-sulfate, and 9% nonsulfate), mouse anti-human glycophorin A and B monoclonal IgG, and 1-propyl gallate were from Sigma Chemical Co., St. Louis, MO. SYBR Green I, 4,6-diamidino-2-phenylindole (DAPI), Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L), and goat anti-rabbit IgG (H + L) were from Molecular Probes, Eugene, OR. Titermax adjuvant was from CytRx Corp, Norcross, GA. Alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L), horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L), and ABTS substrate were from Kirkegaard Perry Laboratories, Gaithersburg, MD. The Vectastain Elite ABC kit (containing biotinylated goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgG, horseradish peroxidase-conjugated avidin, and diaminobenzidine tetrahydrochloride substrate), hematoxylin, eosin, and Methyl Green were from Vector Laboratories, Burlingame, CA. Human blood and serum for parasite culturing were from Hershey Medical Center, Pennsylvania State University, Hershey, PA.

Immunization of Rabbits

The proteoglycans, BCSPG-2 (low-sulfated CSPGs) and DS/CSPG-2 (DS/CSPG), were isolated from the normal

human term placentas and purified by CsBr density gradient centrifugation followed by gel filtration on Sepharose CL-4B as described previously.[44] To prepare preimmune serum, blood was collected from each rabbit before immunization. For the production of anti-CSPG antibody, the animals were immunized with 600 μg of the low-sulfated CSPGs in 300 μl of PBS, pH 7.2, emulsified with 300 μl of Titermax adjuvant. The animals were boosted after 2, 4, 7, 10, and 13 weeks with similar amounts of antigen. For anti-DS/CSPG antibody production, the rabbits were immunized with DS/CSPG (500 μg) in 250 μl of PBS, pH 7.2, emulsified with 250 μl of Titermax. The booster injections were given after 3, 5, and 6 weeks with the similar amounts of DS/CSPG. In both cases, the antibody responses were monitored by enzyme-linked immunosorbent assay. The antibody titers for CSPGs and DS/CSPG were maximal after 15 and 8 weeks of initial immunization, respectively.

Isolation of Rabbit IgG

The IgG from the antisera were isolated by Protein A-Sepharose CL-4B affinity chromatography. Briefly, antisera were diluted with an equal volume of 100 mmol/L Tris-HCl buffer, pH 7.0, and chromatographed on Protein A-Sepharose CL-4B columns. After washing the columns, the bound antibodies were eluted with 0.1 mol/L of glycine, pH 3.0, and the pH adjusted to 7.0. The IgG concentrations were determined by absorption at 280 nm.

Assessment of Rabbit Antisera by Enzyme-Linked Immunosorbent Assay

Ninety-six-well microtiter plates were coated with 50 μl/well of the low-sulfated CSPGs (20 μg/ml) and DS/CSPG (10 μg/ml) in 100 mmol/L bicarbonate buffer, pH 9.0, at 4°C overnight. The wells were then blocked with 150 μl of 0.5% casein in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5, containing 0.05% Tween 20 (TBST) at room temperature for 2 hours. In another set of experiment, the wells coated with proteoglycans were blocked with casein and incubated with chondroitinase ABC (5 mU) in 50 μl of 100 mmol/L Tris-HCl, 30 mmol/L NaOAc, pH 8.0, containing 0.01% bovine serum albumin (BSA) at 37°C for 5 hours.[45] The plates were washed with TBST, and incubated with 50 μl of sera serially diluted with TBST. The bound antibodies were measured using horseradish peroxidase-conjugated goat anti-rabbit IgG and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate.

Chondroitinase ABC Digestion

The purified low-sulfated CSPGs or DS/CSPG (50 μg each) were treated with chondroitinase ABC (20 mU) in 50 μl of 100 mmol/L Tris-HCl, 30 mmol/L NaOAc, pH 8.0, containing 0.01% BSA at 37°C for 5 hours,[45] and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Electrophoresis

SDS-PAGE was performed according to the procedure of Laemmli[46] using 4 to 15% gradient polyacrylamide mini gels under reducing conditions. The gels were stained with Coomassie Blue followed by Alcian Blue and ammoniacal silver.[47]

Western Blotting

The untreated and chondroitinase ABC-treated placental proteoglycans (10 to 15 μg) were electrophoresed as above and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 1% BSA and probed with 10 μg/ml of anti-CSPGs or anti-DS/CSPG antibodies. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development substrates.

Immunohistochemical Localization

Pieces of fresh placentas (1.5 × 1.5 cm) were either snap-frozen by dropping into liquid nitrogen or fixed in PBS, containing the following reagents: 1) 10% formalin at room temperature for 48 hours and stored at 4°C; 2) 2% formalin and 1% cetylpyridinium chloride at room temperature and stored at room temperature; 3) 2% formalin and 0.5% glutaraldehyde for 10 minutes, and then heated in a microwave oven for 60 seconds at a precalibrated power setting so that the solution attains 45°C. The fixed tissue was then transferred to ice-cold PBS, and stored at 4°C.[48,49] The tissues were paraffin-embedded, and cut into 5-μm-thick sections onto glass slides. The frozen tissues were cut into 5-μm-thick sections, air-dried overnight, and fixed with cold acetone at −20°C for 30 minutes. The sections were incubated in an oven at 60°C for 20 minutes.

The paraffin-embedded tissue sections were deparaffinized, and rehydrated by immersion in 100%, 95%, and 70% ethanol, and then in Tris-buffered saline, pH 8.0 (TBS). For enzyme and antibody incubations and for IRBC overlaying, the boundaries of the tissue sections on slides were marked with a wax pen. For histochemical studies, the sections were pretreated with 3% H_2O_2 for 4 minutes, washed with TBS, and blocked with 1:60 diluted normal goat serum in TBS followed by incubation with either 10 μg/ml of the purified anti-proteoglycan antibodies or 1:200 diluted monoclonal antibodies for 60 minutes. Sections incubated with 10 μg/ml of the purified IgG from preimmune sera were used as controls. Tissue sections incubated with chondroitinase ABC (500 mU/ml) in 100 mmol/L Tris-HCl, 30 mmol/L NaOAc, pH 8.0, at 37°C for 3 hours were used to determine antibody reactivity to core proteins and to Δdi-4S and Δdi-6S epitopes. After washing, the slides were incubated for 30 minutes with the biotinylated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-mouse IgM antibodies (Vectastain Elite ABC kit). After washing with TBS, the slides were incubated with horseradish peroxidase-conjugated avidin for

20 minutes, washed again, and then incubated with 0.1% diaminobenzidine tetrahydrochloride and 0.02% H_2O_2 for 5 minutes according to Vectastain Elite ABC kit procedure. The slides were washed with water, counterstained with methyl green, dehydrated, mounted using Permount, and examined under light microscopy.

Selection of C4S-Adherent Parasites

C4S-adherent *P. falciparum* were selected by panning of the 3D7 parasite strain on plastic plates coated with the human placental low-sulfated CSPGs.[38] The parasites (3D7-C4S) were cultured in RPMI 1640 medium using 10% O^+ human serum and type O^+ human red blood cells.[38] Blood from *P. falciparum*-infected placentas were obtained as described.[18]

IRBC Adherence to the Placental Tissue Sections

Sections (5 μm thick) of the fixed placental tissue were deparaffinized, rehydrated, and then blocked with 2% BSA in PBS, at room temperature for 60 minutes. The 3D7-C4S parasites at the midtrophozoite stage (0.1 ml, ~20% parasitemia) were washed with PBS, suspended in 2 ml of PBS, and stained with 1:10,000 diluted SYBR Green at room temperature for 5 minutes by gentle mixing. In the case of parasites from *P. falciparum*-infected placentas (0.1 ml, 10 to 12% parasitemia), the cryopreserved samples were thawed and washed with incomplete medium. The cells were suspended in PBS and stained with SYBR Green as above. In both cases, the cells were washed five times at 20-minute intervals using 15 ml of PBS each time; only IRBCs but not RBCs were found to be stained and emitted green fluorescence. A 2% suspension of the stained parasite culture in 150 to 200 μl of PBS, was overlaid onto the tissue sections, incubated at room temperature for 5 minutes, and washed. For the inhibition of IRBC adhesion, 2% suspensions of the SYBR Green-stained parasite culture were preincubated with 1.25, 5, 20, or 80 μg/ml of bovine tracheal chondroitin sulfate A at room temperature for 30 minutes, and then overlaid onto the tissue sections. To confirm further the C4S-dependent adhesion of IRBCs, the placental tissue sections were treated with chondroitinase ABC (500 mU/ml) in 150 μl of 100 mmol/L Tris-HCl, 30 mmol/L NaOAc, pH 8.0, containing 0.01% BSA at 37°C for 3 hours and performed the assay as above. Tissue sections incubated with the buffers alone were used as controls. To wash the unbound cells, the wax markings on the slides were removed using cotton swabs wetted with acetone. The slides were then placed at slanted positions, and washed by a gentle flow of 6 to 8 ml of PBS from the top of the slides. The slides were mounted using 15 μl of 0.5% 1-propyl gallate and 50% glycerol in PBS, and viewed under fluorescence and light microscope at ×40 resolution.

Localization of Endogenous RBCs in the Placenta and Co-Localization of IRBC Adherence and CSPGs

The placental tissue sections were rehydrated and blocked, and incubated with 10 μg/ml of rabbit anti-CSPG antibodies or 1:400 diluted anti-human glycophorin A and B antibodies as described above. After washing, the sections were incubated with 1:400 diluted Alexa Fluor 568 goat anti-mouse IgGs along with DAPI. After 20 minutes, the slides were washed, and the adherence of IRBC was performed as described above. The slides were mounted and viewed as above.

Results

Production and Characterization of Antibodies Against the Core Proteins of CSPGs and DS/CSPG of Human Placenta

The low-sulfated CSPGs and the tissue-matrix DS/CSPG were purified free of contaminating proteins as described previously.[44] Immunization of rabbits with the low-sulfated CSPGs and tissue matrix DS/CSPG of human placenta produced antisera with significant levels of antibody titer (Figure 1). Enzyme-linked immunosorbent assay analysis of the antisera prepared from the test bleeds showed the presence of significant levels of IgG after the third boost for the low-sulfated CSPGs and after the second boost for DS/CSPG. Continued boosting gradually increased the IgG titer, reaching maximum response at 15 and 8 weeks for the low-sulfated CSPGs and DS/CSPG, respectively (not shown). In both cases, the preimmune sera from the respective animal did not show immunoreactivity with the proteoglycans (Figure 1). The antisera reacted similarly or at slightly higher levels with the chondroitinase ABC-treated proteoglycans compared to the untreated proteoglycans (Figure 1). This clearly demonstrates that the antibodies were directed exclusively against the core proteins of the proteoglycans but not against the ubiquitously expressed glycosaminoglycan chains that are nonimmunogenic. The immunoglobulins in the antisera were purified by Protein A-Sepharose affinity chromatography and used for Western blotting and immunohistochemical analyses (see below).

SDS-PAGE showed a broad high-molecular weight band for the untreated low-sulfated CSPGs, and two distinct >200-kd and ~56-kd core protein bands for the chondroitinase ABC-treated CSPGs (Figure 2A, lanes 1 and 2). These data are in agreement with the results of a previous study that the low-sulfated placental CSPGs have two distinct core proteins, a high- and low-molecular weight species.[44] As observed in the previous study,[44] SDS-PAGE of the chondroitinase ABC-treated DS/CSPG showed 43- to 48-kd protein bands, and the intact proteoglycan showed a 100- to 150-kd broad proteoglycan band (Figure 2A, lanes 3 and 4).

Western blotting was performed to test the specificities of the anti-proteoglycan antibodies. The antibodies

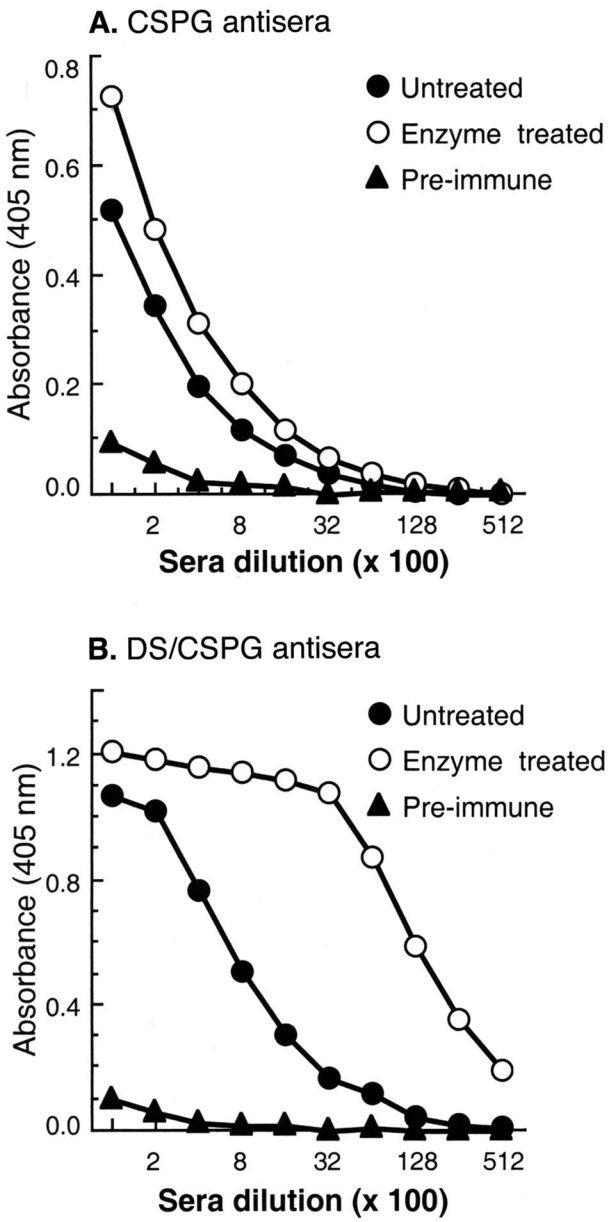


Figure 1. Enzyme-linked immunosorbent assay of antisera against placental proteoglycans. The purified low-sulfated CSPGs and DS/CSPG were coated onto 96-well microtiter plates at 20 μg/ml and 10 μg/ml, respectively. The wells were blocked with 0.5% casein in TBST, pH 7.5, and then incubated with either antisera or preimmune sera serially diluted in TBST containing 0.5% casein. After washing the plates, the bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG using ABTS substrate. The assays were performed two times each in duplicates, and average OD values plotted. **A:** Antisera against the placental low-sulfated CSPGs before (●) and after (○) treatment of the CSPGs with chondroitinase ABC, and preimmune sera after treatment of the low-sulfated CSPGs with chondroitinase ABC (▲). **B:** Antisera against DS/CSPG before (●) and after (○) treatment of DS/CSPG with chondroitinase ABC, and preimmune sera after treatment of DS/CSPG with chondroitinase ABC (▲). In both cases the reactivities of preimmune sera before treatment with chondroitinase ABC were also at the background levels (not shown).

against the low-sulfated placental CSPGs specifically reacted with the high (>200 kd)- and low (~56 kd)-molecular size core proteins of the proteoglycans; the 43- to 48-kd core proteins of the placental tissue matrix DS/CSPG were nonreactive (Figure 2B, lanes 1 to 4). Conversely, the antibodies against the placental DS/CSPG specifically reacted with the 43- to 48-kd core proteins of the DS/CSPG, but not with the core proteins of the low-sulfated CSPGs (Figure 2C, lanes 1 to 4). In both cases, the IgGs isolated from the preimmune sera of the respective animals did not react with either the intact proteoglycans or their core proteins (data not shown). These data confirm that the antibodies were directed against the core proteins of respective proteoglycan. The observed lower level of reactivity of the intact proteoglycans compared to the released core proteins could be because of the low efficiency of transfer of the intact proteoglycans from the polyacrylamide gels onto polyvinylidene difluoride membranes.

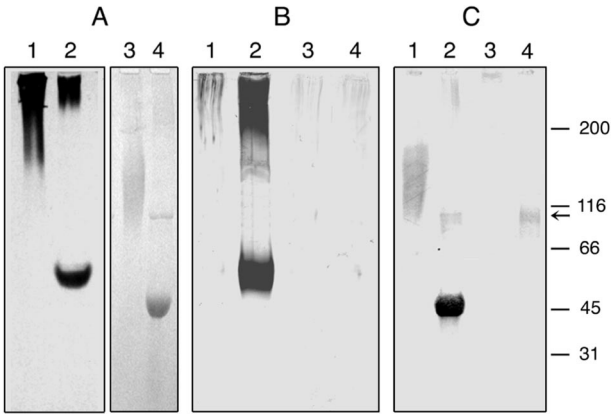


Figure 2. SDS-PAGE and Western blot analysis of the low-sulfated CSPGs and DS/CSPG purified from human placenta. **A:** Alcian Blue followed by silver staining of 4 to 15% gradient gel electrophoresed with the purified low-sulfated CSPGs and tissue matrix DS/CSPG before (**lanes 1** and **3**, respectively) and after (**lanes 2** and **4**, respectively) treatment with chondroitinase ABC. **B:** Immunoblots of the low-sulfated CSPGs and DS/CSPG before (**lanes 1** and **3**, respectively) and after (**lanes 2** and **4**, respectively) treatment with chondroitinase ABC using antibodies against the placental low-sulfated CSPGs. **C:** Immunoblots of the DS/CSPG and the low-sulfated CSPGs before (**lanes 1** and **3**, respectively) and after (**lanes 2** and **4**, respectively) treatment with chondroitinase ABC using antibodies specific to DS/CSPG. In all of the panels, each lane was loaded with 15 μg of proteoglycans. The positions (kd) of the molecular mass marker proteins are indicated on the **right**. Note: The **arrow** on the **right** indicates the position of chondroitinase ABC; the immunoreactivity of which is most likely because of the natural exposure of rabbits to the enzyme-producing bacteria.

Immunohistochemical Localization of the Low-Sulfated CSPGs and DS/CSPG in the Placenta

Localization of the major CSPG types present in human placenta was performed by immunohistochemical analysis using the antibodies against the low-sulfated CSPGs and DS/CSPGs and the monoclonal antibodies that are specific to the unsaturated disaccharide motifs, Δdi-4S and Δdi-6S, formed on core proteins by the action of chondroitinase ABC on CSPGs and DS/CSPG.[50,51] Chondroitinase ABC degrades the disaccharide repeats of the glycosaminoglycan chains of CSPGs or DSPGs, releasing Δdi-0S, Δdi-4S, and Δdi-6S disaccharides but leaves one unsaturated disaccharide moiety at the linkage regions of the chondroitin sulfate or dermatan sulfate chains on the core proteins. Therefore, after chondroitinase ABC treatment, there will be one unsaturated di-

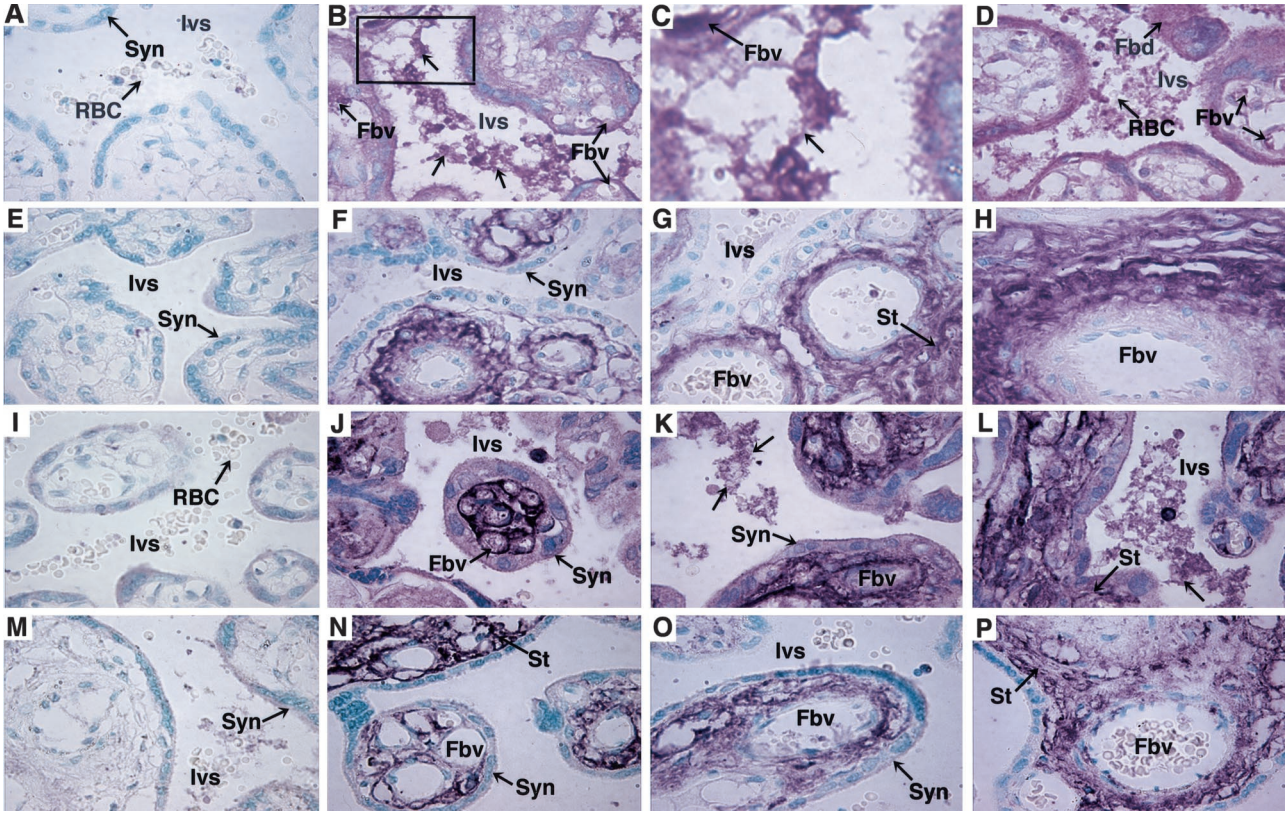


Figure 3. Immunohistochemical localization of the CSPGs of human placenta. The placental tissue sections were immunostained with either polyclonal antibodies against the placental proteoglycan core proteins (**B** to **D** and **F** to **H**) or monoclonal antibodies specific to the Δdi-4S (**I** to **L**) and-Δdi-6S (**M** to **P**) moieties in the core proteins formed by the chondroitinase ABC treatment of the proteoglycans. **A:** Only background level of staining was observed with the preimmune serum from the rabbit used for raising antibodies against the placental low-sulfated CSPGs. **B** to **D:** Antibodies against the low-sulfated CSPGs stained fibrous, filamentous materials and fibrinoid deposits in the intervillous space. The fibrous projections from the syncytiotrophoblast layers were also stained. **E:** Preimmune serum from the rabbit used for raising antibodies against DS/CSPG showed no staining. **F** to **H:** Antibodies against the placental DS/CSPG stained strongly the perivascular regions of blood vessels and stroma. **I:** Anti-Δdi-4S monoclonal antibody did not stain tissue sections not treated with chondroitinase ABC. **J** to **L:** Anti-Δdi-4S monoclonal IgG moderately stained the matrix-like material of the intervillous space and strongly stained the perivascular regions of blood vessels, extending to the stroma in the chondroitinase ABC-treated tissue sections. **M:** Anti-Δdi-6S monoclonal antibody did not stain the tissue sections not treated with chondroitinase ABC. **N** to **P:** Anti-Δdi-6S monoclonal IgM stained strongly the stroma in the chondroitinase ABC-treated tissue sections. **C** represents the enlarged area marked in **B**. Ivs, intervillous space; Syn, syncytiotrophoblasts; RBC, red blood cells; Fbd, fibrinoid deposits; Fbv, fetal blood vessel; St, stromal tissue. **Arrows** indicate the staining of the fibrous filament-like materials in the intervillous space. Original magnifications, ×100 (**A**, **B**, **D–P**).

saccharide moiety (Δdi-0S, Δdi-4S, or Δdi-6S) per glycosaminoglycan chain attached to core proteins. Because the low-sulfated CSPGs contain six to eight glycosaminoglycan chains per molecule,[44] six to eight unsaturated disaccharide stubs with Δdi-0S (major) and Δdi-4S (minor) are expected on the core protein after the action of chondroitinase ABC. In the case of placental DS/CSPG, after chondroitinase ABC treatment, there should be only one unsaturated disaccharide moiety per core protein molecule because this proteoglycan has only one glycosaminoglycan chain.[44] Based on the glycosaminoglycan composition, the chondroitinase ABC-treated placental DS/CSPG should have mainly Δdi-6S with moderate amount of Δdi-4S and low amounts of Δdi-0S.[44] Although the rabbit polyclonal antibodies will localize the placental CSPGs based on their core protein identity, the anti-Δdi-4S and anti-Δdi-6S monoclonal antibodies will localize the proteoglycans based on the sulfation type in the glycosaminoglycan chains.[50,51] Initial studies indicated that the intervillous space materials are not preserved in the snap-frozen placental tissues, which is in agreement with previous reports (not shown).[26,42] Therefore, pilot studies were done using tissues fixed by different procedures. It was found that, in all of the cases, except fixing followed by heating by microwave, most of the intervillous space materials were either lost or tissue sections were not suitable for adherence assay (not shown). Therefore, we used tissue sections fixed with formalin and glutaraldehyde followed by microwave treatment for immunohistochemical localization of the CSPGs and for the IRBC adherence assays.

The antibodies that are specific to the core proteins of the low-sulfated CSPGs stained the fibrous and filament-like structures (Figure 3, B and C, arrows), and fibrinoid deposits in the intervillous space (Figure 3D) that represent extracellular materials containing the low-sulfated CSPGs. The antibodies also stained the fibrous-like projections from the syncytiotrophoblasts (Figure 3, B and C). Syncytiotrophoblasts were also stained moderately, suggesting that the CSPGs are synthesized by the trophoblasts. The antibodies isolated from the preimmune serum, showed only background levels of staining (Figure 3A), confirming that the staining in the intervillous space is specific for the low-sulfated CSPGs.

In contrast to the antibodies against the low-sulfated CSPGs, no staining was observed in the intervillous space with the anti-DS/CSPG antibodies (Figure 3; F to H). However, the latter antibodies strongly stained perivascular regions of the blood vessels and the stromal tissue (Figure 3; F to H). These results indicate that the DS/CSPG, as expected, is present in the tissue matrix as extracellular molecules embedded in the collagen meshwork.[52] The antibodies from the preimmune serum did not stain the tissue (Figure 3E), demonstrating that the reactivity was because of specific antibodies against DS/CSPG at these locations.

The antibodies against the placental low-sulfated CSPGs and tissue-matrix DS/CSPGs were also tested using chondroitinase ABC-treated tissue sections. Consistent with the immunoreactivity of these antibodies against the core proteins of the respective proteoglycans, in both cases, the staining intensities were comparable to those of untreated tissue sections (data not shown).

Both the anti-Δdi-4S and anti-Δdi-6S monoclonal antibodies showed strong staining of the perivascular regions of blood vessels and the stroma (Figure 3; J, K, L, and N, O, P, respectively). These results are consistent with the high abundance of DS/CSPG at these locations; the DS/CS chains can yield high levels of Δdi-4S and Δdi-6S epitopes on treatment with chondroitinase ABC. The anti-Δdi-4S antibody but not anti-Δdi-6S antibody also stained the intervillous space material (Figure 3; J to L), indicating the absence of 6-sulfated CS chains in the placental intervillous space. However the staining by the anti-Δdi-4S antibody in the intervillous space was only moderate despite the presence of high levels of CSPGs at this location. This is because of the very low level of 4-sulfate (on an average only ~8%) in these CSPGs[44] that will give rise to only a few Δdi-4S epitopes on the core proteins after chondroitinase ABC treatment. Consistent with the reactivity toward Δdi-4S and Δdi-6S epitopes, the monoclonal antibodies did not stain the tissue sections not treated with chondroitinase ABC (Figure 3, I and M, respectively).

IRBC Adherence to the Human Placental Tissue Sections

If the low-sulfated CSPGs in the intervillous space of the placenta are the receptors for the IRBC adherence, then IRBCs should bind densely at these sites in a C4S-dependent manner. To investigate this, we initially examined the adhesion of IRBCs to the sections of snap-frozen and formalin-glutaraldehyde-fixed tissues using 3D7 parasites. IRBCs were not bound in the intervillous space of the snap-frozen sections (probably because of the loss of matrix-like material containing the low-sulfated CSPGs), but bound only to the syncytiotrophoblasts (not shown) similar to that reported previously.[26,42] However, when microwave-treated tissue sections with the intervillous space materials preserved were used, IRBCs bound abundantly (see below).

For IRBC adherence assay with the fixed tissue sections, the IRBCs were stained with SYBR Green (DAPI also works) before overlaying onto the tissue sections. After incubation and washing to remove nonadherent IRBCs and RBCs, the tissue sections were viewed under fluorescent microscope. Because in this assay, only IRBCs but not RBCs from the 3D7-C4S culture or placental blood samples were bound to the tissue sections, counting and scoring of bound IRBCs were judged to be of no relevance. Approximately 50 tissue sections from two different placentas were studied using 3D7-C4S parasites. The IRBC binding pattern in all tissue sections were similar, and data from two representative tissue sections are presented. IRBCs adhered at high density in the intervillous space and on some of the syncytiotrophoblasts (Figure 4, A to D, and Figure 5). IRBCs were not seen in the empty space, the areas of the intervillous space where matrix components were absent, or on the stromal tissue (Figure 4, A to D, and Figure 5). To show that, *in vivo*, IRBCs adhere predominantly in the intervillous space, we tested blood samples collected from placentas of two *P. falciparum*-infected Cameroonian women using 10 different tissue sections. In all cases, IRBC adherence patterns (Figure 4; E to H) were similar to those observed for 3D7-C4S parasites (Figure 4; A to D).

The dense binding of IRBCs in certain areas of the intervillous space is because of the marked shrinkage of the matrix-like material by the fixatives used for the tissue fixing, resulting in the localized high concentrations of CSPGs (Figure 5). This explains why IRBCs are not adhered in some areas of the intervillous space. When the tissue sections were treated with chondroitinase ABC to degrade the C4S chains, the adherence of IRBCs was abolished by ~98% (Figure 4, I and J). IRBC adherence to the tissue sections was inhibited in a dose-dependent manner when the parasite cultures or placental blood samples were preincubated with C4S before overlaying; at 80 μg/ml, the inhibition was ~99% (Figure 4, K and L). At 20, 5, and 1.25 μg/ml, C4S inhibited IRBC adhesion by 90%, 80%, and 55%, respectively (not shown). Consistent with the previous reports that C4S- but not CD36-adherent parasites sequester in human placenta,[34] the latter parasites were unable to bind to the placental tissue sections (not shown). Because the antibodies against placental proteoglycans were directed against core proteins, they were not examined for IRBC adhesion inhibition.

A comparison of the fluorescent and light microscopy patterns of tissue sections showed some extra RBCs (indicated by arrowheads) in the tissue sections (compare Figure 4, A with B; C with D; E with F; G with H). In Figure 4, I and J, although only very few IRBCs were adhered, a significant number of RBCs are present (Figure 5). These RBCs are those already present in the fixed tissue sections. These data, together with those described above demonstrate the C4S-dependent binding of IRBCs, ruling out the nonspecific binding of IRBCs and RBCs (Figure 5). The IRBCs were not adhered in the stromal tissue, which is consistent with the low adherence capacities of the tissue matrix DS/CSPG bearing GAG chains with predominantly 6-sulfated disaccharide moieties.[44] Unexpectedly, however, IRBCs were bound

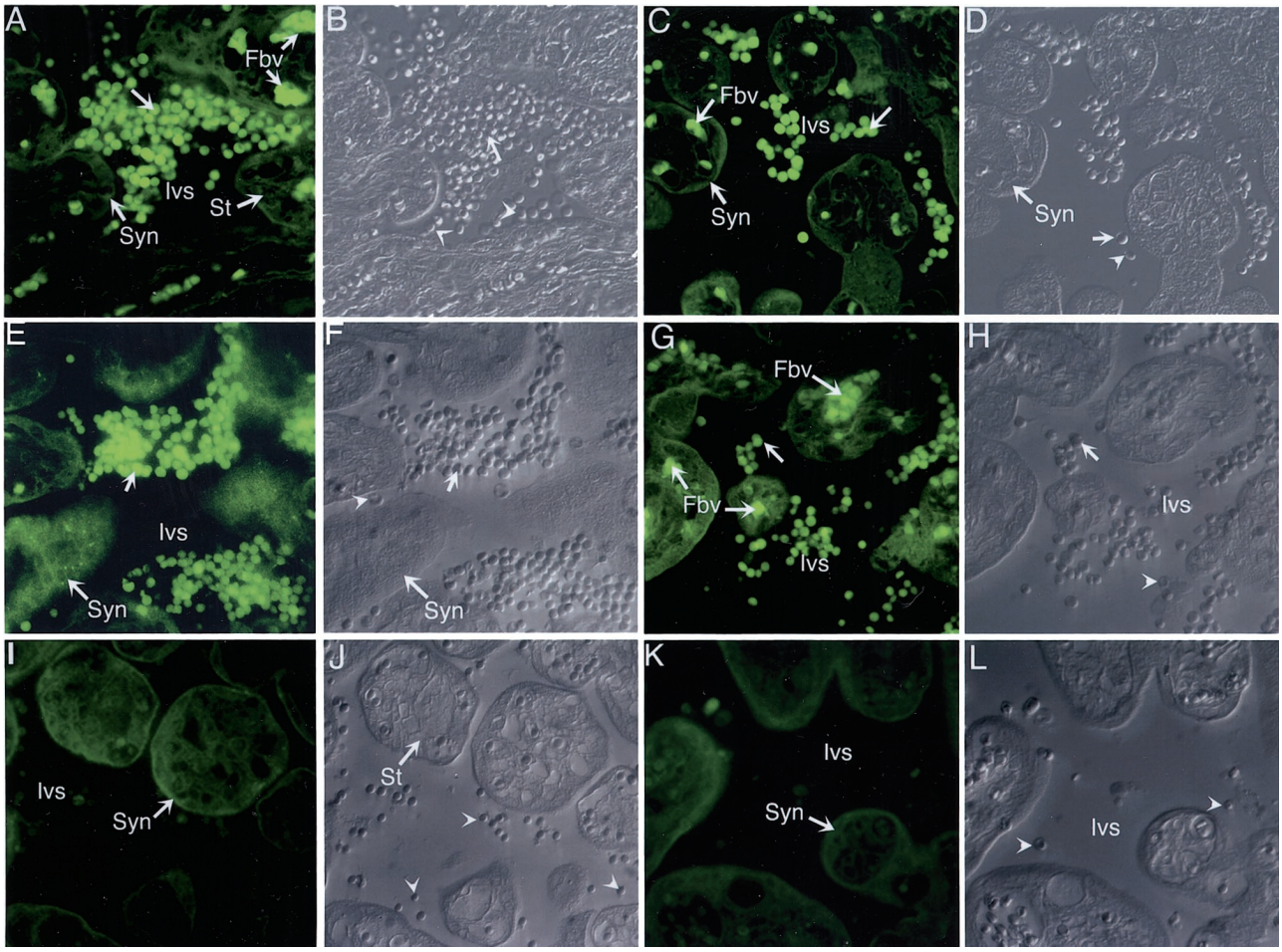


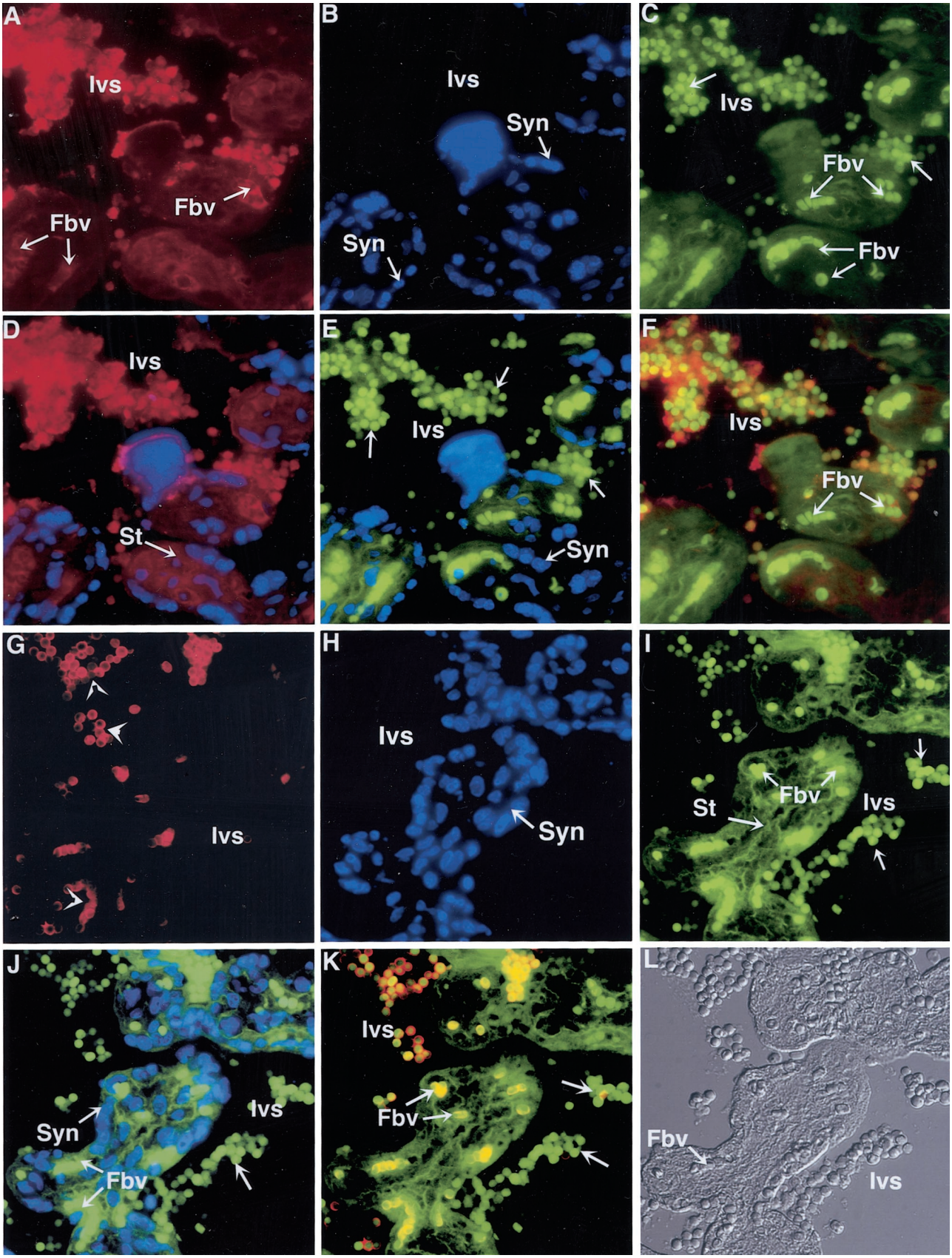
Figure 4. *Ex vivo* adherence of *P. falciparum* IRBCs to human placenta. The 3D7-C4S parasites or placental blood samples were prestained with SYBR Green and overlaid onto the placental tissue sections fixed with formaldehyde and glutaraldehyde followed by microwave treatment. The unbound IRBCs and RBCs were washed, and the sections were examined and photographed under fluorescence microscopy (**A**, **C**, **E**, **G**, **I**, and **K**) and light microscopy (**B**, **D**, **F**, **H**, **J**, and **L**). **A** and **C:** Fluorescent micrographs of the tissue sections showing SYBR Green-stained 3D7-C4S IRBCs densely adhered in the intervillous space and moderately adhered to the syncytiotrophoblasts. **B** and **D:** Light micrographs of fields corresponding to **A** and **C**, respectively. **E** and **G:** Fluorescent micrographs of the tissue sections showing SYBR Green-stained placental IRBCs adhered in the intervillous space and to syncytiotrophoblast cell layer. **F** and **H:** Light micrographs of fields corresponding to **E** and **G**, respectively. **I:** Fluorescent micrograph of the chondroitinase ABC-treated tissue sections overlaid with the SYBR Green-stained IRBCs. **J:** Light micrograph of the field corresponding to **I**; the RBCs (**arrowhead**) present in **J** were because of those present in the fixed tissue section (also see Figure 5). **K:** Fluorescent micrograph of the tissue sections overlaid with the SYBR Green-stained IRBCs preincubated with 80 μg/ml of C4S. **L:** Light micrograph of the field corresponding to **K**; as in **J**, the observed RBCs (**arrowhead**) were because of those present in the fixed tissue section (also see Figure 5). Ivs, intervillous space; Syn, syncytiotrophoblasts; Fbv, fetal blood vessels; St, stromal tissue. **Arrows** indicate the IRBCs; **arrowheads** indicate the RBCs. Original magnifications, ×40.

densely also in the blood vessels of the fetal villi, suggesting the presence of a CSPG at this location that efficiently binds IRBCs (Figure 4, A and G, and Figure 5). Further studies are needed to determine the relationship of this CSPG to the low-sulfated CSPGs in the intervillous space.

Because of the dense adherence of IRBCs in the intervillous space and possible disruption of the intervillous material because of mechanical compression during delivery and handling of placenta, it was not considered realistic to obtain accurate counting and thereby calculate proportions of IRBCs that could adhere at various locations of the placenta. However, a semiquantitative counting revealed that IRBCs adhered in ~9:1 ratio in the intervillous space and on syncytiotrophoblasts.

In all of the tissue sections used for IRBC adherence assay using SYBR Green-stained IRBCs, the syncytiotrophoblasts were also significantly stained (Figures 4 and 5). This was because of the slow release of SYBR Green by the stained IRBCs during adherence assay, and it was

Figure 5. Co-localization of the low-sulfated CSPGs and IRBC adherence in the placenta, and dual-fluorescence staining of syncytiotrophoblasts and endogenous RBCs in the placenta. Placental tissue sections were stained with DAPI and anti-CSPG or anti-glycophorin A and B antibodies, and then adherence with SYBR Green-stained IRBCs performed as described in Figure 4. **A** to **C:** Fluorescent micrographs of the tissue sections stained with the antibodies against the placental low-sulfated CSPGs (**A**) and with DAPI (**B**) followed by adherence with SYBR Green-stained IRBCs (**C**). **D** to **F:** Merged pictures of **A** and **B** (**D**), **B** and **C** (**E**), and **A** and **C** (**F**). **G** to **I:** Fluorescent micrograph of the tissue sections stained with the monoclonal antibodies specific to human glycophorin A and B (**G**) and with DAPI (**H**) followed by adherence with SYBR Green-stained IRBCs (**I**). **J** and **K:** Merged pictures of **H** and **I** (**J**) and **G** and **I** (**K**). **L:** Light micrograph of tissue sections shown in **G** to **K**. Ivs, intervillous space; Syn, syncytiotrophoblasts; Fbv, fetal blood vessels; St, stromal tissue. **Arrows** indicate the IRBCs; **arrowheads** indicate the RBCs. Original magnifications, ×40.


A
Ivs
Fbv
Fbv
B
Ivs
Syn
Syn
C
Ivs
Fbv
Fbv
D
Ivs
St
E
Ivs
Syn
F
Ivs
Fbv
G
Ivs
H
Ivs
Syn
I
St
Fbv
Ivs
J
Syn
Ivs
Fbv
K
Ivs
Fbv
L
Fbv
Ivs

persisted despite washing IRBCs repeatedly throughout a longer period of time. However, it should be noted that this staining does not interfere with the results expected from these experiments.

Dual-fluorescence staining was done to determine the co-localization of IRBCs and placental CSPG receptor and to demonstrate that the RBCs present in the tissue sections are endogenous. When incubated with the rabbit polyclonal anti-CSPG antibodies and probed with Alexa Fluor 568-conjugated secondary antibody, the tissue sections exhibited a strong red fluorescence in the intervillous space, indicating that the low-sulfated placental CSPGs are predominantly localized in the intervillous space and the fetal blood vessels (Figure 5A). The staining of the same tissue sections with DAPI (to localize the syncytiotrophoblasts) and the adherence of SYBR Green-stained IRBCs are shown in Figure 5, B and C, respectively. Anti-CSPG antibodies strongly stained the intervillous space and fetal blood vessels. SYBR Green-stained IRBCs adhered predominantly in the intervillous space and in the fetal blood vessel, where CSPG is concentrated. A merge of fluorescent micrographs Figure 5, A and B, clearly distinguishes the syncytiotrophoblast surface and intervillous space (Figure 5D). Whereas, the merge of DAPI staining and IRBC-adhered fluorescent micrographs, Figure 5, B and C, indicates the binding of IRBCs predominantly in the intervillous space and in the fetal blood vessels and some IRBC adherence to the syncytiotrophoblasts (Figure 5; E, I, and J). Further, a merge of the CSPG-stained and IRBCs adhered fluorescent micrographs Figure 5, A and C, demonstrated the co-localization of the adhered IRBCs and the CSPGs in the intervillous space (Figure 5F). Similarly, staining of the placental tissue sections with anti-glycophorin A and B antibodies revealed the presence of endogenous RBCs (Figure 5G). The same tissue section stained with DAPI followed by IRBC adherence are shown in Figure 5, H and I, respectively. A merge of the fluorescent micrographs Figure 5, H and I, showed the presence of endogenous RBCs in the intervillous space and fetal blood vessels, where the IRBCs adhered to the CSPGs (Figure 5J). A merge of micrographs Figure 5, G and I, clearly demonstrates the presence of IRBC association with the intervillous space material and co-localization of adhered IRBCs (Figure 5K). Further, a comparison of fluorescent micrograph Figure 5, G and I, with the light micrograph Figure 5L indicates the absence of exogenous uninfected RBCs and the exclusive presence of endogenous RBCs in the IRBC adhered tissue sections (Figure 5L). Thus, collectively, the data presented in Figure 5, A to L, unequivocally establish the localization of the low-sulfated CSPGs predominantly in the intervillous space and at low but significant levels on the syncytiotrophoblasts. The data also establish that the C4S-dependent binding of IRBCs to the low-sulfated CSPGs, and that these proteoglycans are the predominant, if not exclusive, receptors for IRBC adherence in the placenta. IRBCs also adhered densely in the fetal blood vessels, and the adherence co-localized with the CSPGs present at high concentrations at these locations as revealed by immunofluorescence analysis using antibodies against the low-sulfated CSPGs (Figure 4, A and G; Figure 5, A to F).

IRBC Adherence in P. falciparum*-Infected Placentas*

We next examined IRBC adherence pattern in the infected placentas to determine whether the observed *ex vivo* IRBCs adherence pattern resemble that of the natural adherence. The *in vivo* IRBC adherence patterns in four different placentas with 16%, 41%, 46%, and 50% placental parasitemia are shown in Figure 6. In all four placentas, IRBCs are present mainly in the intervillous space; but significant IRBC adherence to syncytiotrophoblast lining is also evident (Figure 6). As reported previously,[26,40,41] many IRBCs are surrounded by RBCs and thus are not accessible for direct binding to the syncytiotrophoblast surface. However, a significant number of cells are lined against the syncytiotrophoblasts and many of these seem to have attached to the cells. This adherence pattern closely resembles the distribution pattern of the low-sulfated CSPGs in the placenta, ie, in the intervillous space as well as the fibrous projections from the syncytiotrophoblasts. Furthermore, in agreement with the strong staining of the fibrinoid deposits by the antibodies against the low-sulfated CSPGs, IRBCs bound densely on the surfaces of these deposits (Figure 6D).

Discussion

In this study, we developed an *ex vivo* adherence analysis using placental tissue sections fixed with formalin and glutaraldehyde followed by microwave treatment that enhanced the preservation of the intervillous space materials. Using this assay, we show that *P. falciparum* IRBCs can bind at high densities in the intervillous space but at low levels on the syncytiotrophoblasts of the placenta in a C4S-dependent manner. The *ex vivo* adherence pattern closely resembles the pattern of IRBC adherence observed in placentas that are highly infected with *P. falciparum*, that is dense adherence of IRBCs in the intervillous space and sparingly on the syncytiotrophoblasts. These *ex vivo* and *in vivo* IRBC adherence patterns paralleled the localization patterns of the low-sulfated CSPGs of the placenta, that is, mainly in the intervillous space and at low levels on the syncytiotrophoblasts. Furthermore, our data clearly demonstrate that the low-sulfated CSPGs are the only proteoglycan type present in the intervillous space. In contrast, DS/CSPG, the other major proteoglycan is localized primarily in the perivascular regions of the blood vessels and in the stromal tissues of the placenta, but not in the intervillous space or on the syncytiotrophoblast cells. Together, these findings unequivocally establish that in *P. falciparum-infected* placentas, the IRBCs adhere mainly in the intervillous space and at low but significant levels on the syncytiotrophoblasts.

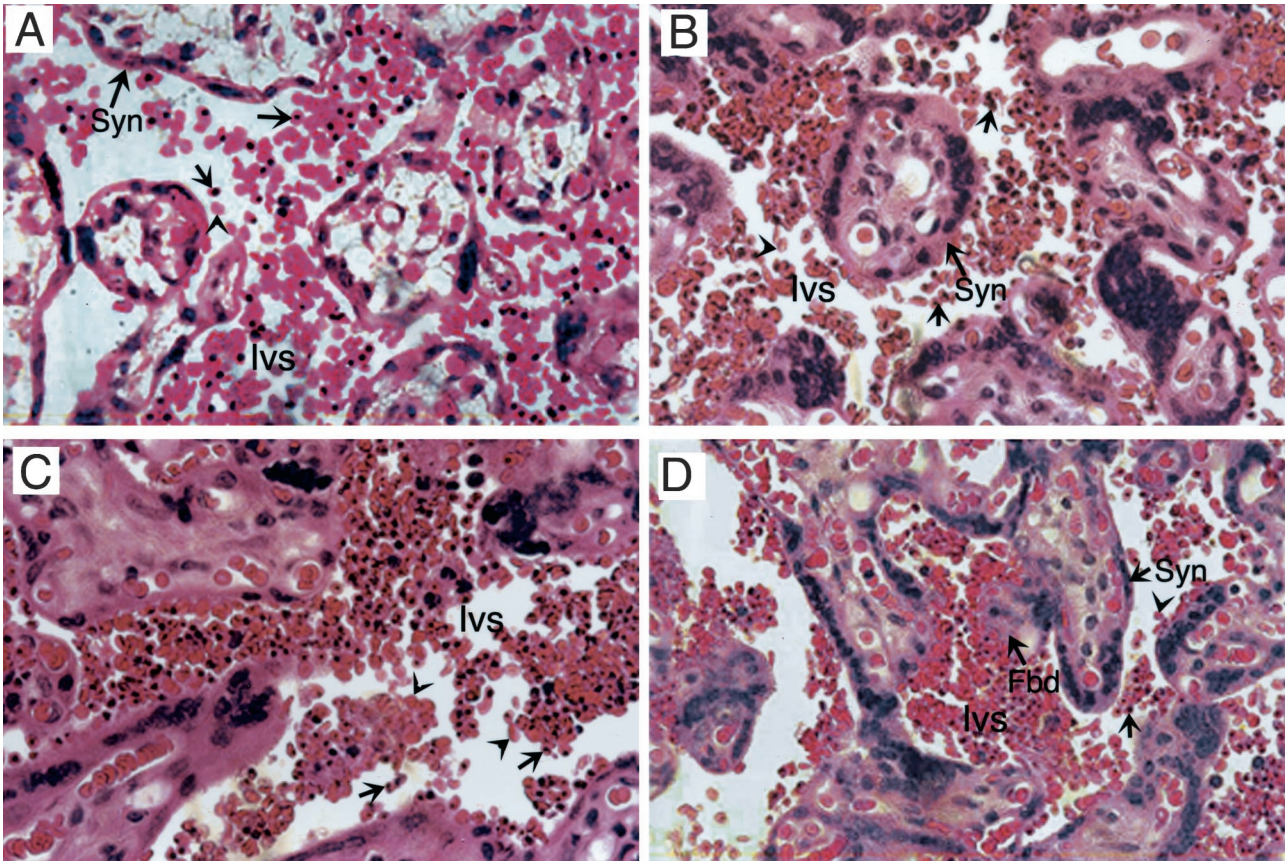


Figure 6. Adherence of IRBCs in *P. falciparum*-infected human placenta. The *P. falciparum*-infected placental tissue was fixed with 10% neutral buffered formalin, sectioned, stained with H&E, and photographed under light microscopy. Massive adherence of IRBCs (those RBCs with stained parasite inside) in the intervillous space, as well as significant level of adherence to syncytiotrophoblast lining in four different infected placentas (**A** to **D**) with 16% (**A**), 41% (**B**), 46% (**C**), and 50% (**D**) placental parasitemia, respectively. **Arrows** indicate the IRBCs; **arrowheads** indicate the RBCs. Original magnifications, ×40.

The following evidence demonstrates that the low-sulfated CSPGs are localized primarily in the intervillous space and at low levels on the syncytiotrophoblasts. 1) The immunostaining pattern by the polyclonal antibodies against the core proteins of the low-sulfated CSPGs. As shown in Figure 3, the antibody staining patterns include fibrous hair-like projections from the syncytiotrophoblasts, and filament-like structures and fibrinoid deposits in the intervillous space. The hair-like projections represent the portions of the loose networks of extracellular matrix materials containing the low-sulfated CSPGs that were bound to syncytiotrophoblasts. 2) The staining of the intervillous space by the monoclonal antibody specific to the Δdi-4S epitopes. The moderate rather than the expected intense staining in the intervillous space by this antibody, despite the presence of the high levels of CSPG at this location, is because of the low proportions of 4-sulfated disaccharide moieties in the CS chains of the CSPGs.[44] 3) The lack of staining by the anti-Δdi-6S monoclonal antibody in the intervillous space, which is consistent with the presence of CSPGs bearing CS chains containing 4-sulfated but not 6-sulfated disaccharide moieties in the intervillous space.

The data presented in here unequivocally show that the low-sulfated CSPGs are the only CSPGs present in the intervillous space, and that the IRBCs adhere mainly in the intervillous space and at low levels on syncytiotrophoblasts in a C4S-dependent manner. The results of dual-fluorescence labeling of the tissue sections using DAPI and anti-CSPG antibodies distinctively revealed the locations of the syncytiotrophoblast lining and the intervillous space where the low-sulfated CSPGs are concentrated. The presence of the low-sulfated CSPG predominantly in certain areas and absence in other areas of intervillous space is likely because of disruption of matrix-like material and coagulation of blood. The co-localization of endogenous RBCs and the CSPGs supports this observation. Further, the co-localization of the densely adhered IRBCs and staining by the antibodies against the low-sulfated CSPGs provide compelling evidence that the low-sulfated CSPGs are the major natural receptors for placental IRBC adherence.

As indicated by the results of immunohistochemical analysis, the low-sulfated CSPGs are also present in minor proportion on the syncytiotrophoblasts either as adsorbed molecules or as molecules in transit to being secreted to the intervillous space. Because the low-sulfated CSPGs belong to aggrecan family extracellular CSPGs,[44] it is unlikely that they are the intrinsic plasma membrane components. Previous studies have shown that the syncytiotrophoblast surface contains thrombomodulin, a part time CSPG.[53,54] Because the abundance

of thrombomodulin is quite low and only ~10 to 20% of the expressed thrombomodulin bears CS chains,[54] the CSPG form of thrombomodulin is expected to be very low. Based on the results of our previous study,[44] other low abundance CSPGs present on the syncytiotrophoblast surface are also likely to carry partially 4-sulfated CS chains. Because IRBCs bind more avidly to partially sulfated C4S rather than the fully sulfated C4S,[38,55] the binding of IRBCs to syncytiotrophoblasts is not surprising. However, based on the results of the previous study by Achur and colleagues,[44] the amount of the low-sulfated CSPGs present extracellularly is approximately an order of magnitude higher than that on the cell surface. Thus, it is expected that the proportion of IRBC binding to the villous surface to be markedly less compared to that of the intervillous space. Consistent with this prediction, the IRBCs that adhered to the intervillous space were approximately ninefold higher in number than those bound to the syncytiotrophoblasts.

The CSPGs with core proteins immunologically similar to those of the low-sulfated CSPGs of the intervillous space are also present at high concentration in the blood vessels of the fetal villi. Therefore, it is not clear, from the results of this study, whether the CSPGs present in the blood vessels of the fetal villi and those of the placental intervillous space are chemically identical and of common origin. Based on the core protein similarity, the dense adherence of IRBCs in the intervillous space and blood vessels of the fetal villi suggests the presence of common low-sulfated CSPGs at these two locations. If the CSPGs at these two locations are indeed biochemically identical, then our data suggest that the CSPGs can cross the placental barrier regardless of whether they are of fetal or maternal origin.

Another aspect of this study is that the *ex vivo* IRBC adherence assay described here will allow for the assessment of IRBC adherence pattern in the placenta and the localization of proteoglycans in the intervillous space. For the success of the assay, the following modifications to the routinely used tissue adherence procedure are crucial: fixing of the placental tissue with formalin and glutaraldehyde followed by treatment with microwave to enhance the preservation of the fragile matrix-like materials in the intervillous space; and prestaining of IRBCs with a fluorescent dye to directly visualize the IRBCs bound to the tissue sections. This step avoids the post-IRBC adhesion fixing and staining steps, which otherwise detach the bound IRBCs because they cannot be cross-linked to the prefixed tissue sections because of the nonavailability of functional groups in the tissue sections. Thus, we were able to successfully demonstrate that the low-sulfated CSPGs are the natural receptors for IRBC adherence and that they are localized in the intervillous space of the placenta. Additionally, because the *ex vivo* IRBC adherence closely resembles the IRBC adhesion pattern in *P. falciparum*-infected placentas, the procedure offers a reliable method for the evaluation and validation of C4S inhibitors and IRBC adhesion inhibitory antibodies in studies aimed at developing therapeutics and/or vaccine for placental malaria.

References

1. Trigg T, Kondrachine AV: The current global malaria situation. Malaria—Parasite Biology, Pathogenesis, and Protection. Edited by IW Sherman. Washington DC, ASM Press, 1998, pp 11–22
2. Snow RW, Craig M, Diechmann U, Marsh K: Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull WHO 1999, 77:624–640
3. Greenwood B, Mutabingwa T: Malaria in 2002. Nature 2002, 415: 670–672
4. Miller LH, Baruch DI, Marsh K, Doumbo OK: The pathogenic basis of malaria. Nature 2002, 415:673–679
5. Pasloske BL, Howard RJ: Malaria, the red cell, and the endothelium. Annu Rev Med 1994, 45:283–295
6. Baruch DI, Gormely JA, Ma C, Howard RJ, Pasloske BL: P. falciparum erythrocyte membrane protein is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule-1. Proc Natl Acad Sci USA 1996, 93:3497–3502
7. Weatherall DJ, Miller LH, Baruch DI, Marsh K, Doumbo OK, Casals-Pascual C, Roberts DJ: Malaria and the red cell. Hematology (American Society of Hematology Education Program Book). Edited by Broudy VC, Abkowitz JL, Vose JM. Washington, DC, Press ASH, 2002, pp 35–57
8. Heddini A: Malaria pathogenesis: a jigsaw with an increasing number of pieces. Int J Parasitol 2002, 32:1587–1598
9. Xiao L, Yang C, Dorovini-Zis K, Tendon NN, Ades ED, Lal AA, Udhayakumar V: Plasmodium falciparum: involvement of additional receptors in the cytoadherence of infected erythrocytes to microvascular endothelial cells. Exp Parasitol 1996, 84:42–55
10. Buffet PA, Gamain B, Scheidig C, Baruch D, Smith JD, Hernandez-Rivas R, Pouvelle B, Oishi S, Fujii N, Fusai T, Parzy D, Miller LH, Gysin J, Scherf A: Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. Proc Natl Acad Sci USA 1999, 96:12743–12748
11. Gamain B, Gratepanche S, Miller LH, Baruch DI: Molecular basis for the dichotomy in Plasmodium falciparum adhesion to CD36 and chondroitin sulfate A. Proc Natl Acad Sci USA 2002, 99:10020–10024
12. Heddini A, Chen Q, Obiero J, Kai O, Fernandez V, Marsh K, Muller WA, Wahlgren M: Binding of Plasmodium falciparum-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): frequent recognition by clinical isolates. Am J Trop Med Hyg 2001, 65:47–51
13. Rogerson SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV: Chondroitin sulfate A is a cell surface receptor for P. falciparum-infected erythrocytes. J Exp Med 1995, 182:15–20
14. Robert C, Pouvelle B, Meyer P, Muanza K, Fujioka H, Aikawa M, Scherf A, Gysin J: Chondroitin-4-sulphate (proteoglycan), a receptor for Plasmodium falciparum-infected erythrocyte adherence on brain microvascular endothelial cells. Res Immunol 1995, 146:383–393
15. Chen Q, Heddini A, Barragan A, Fernandez V, Pearce SF, Wahlgren M: The semiconserved head structure of Plasmodium falciparum erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. J Exp Med 2000, 192:1–10
16. Baird JK: Host age as a determinant of naturally acquired immunity to Plasmodium falciparum malaria. Parasitol Today 1995, 11:105–111
17. Riley EM, Hviid L, Theander TG: Malaria. Parasite Infections and the Immune System. Edited by F Kierszenbaum. New York, Academic Press, 1993, pp 119–143
18. Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalamala F, Rogerson SJ: Plasmodium falciparum isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis 1999, 180:464–472
19. Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M, Marsh K: Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. Infect Immun 2001, 69:5849–5856
20. Beeson JG, Reeder JC, Rogerson SJ, Brown GV: Parasite adhesion and immune evasion in placental malaria. Trends Parasitol 2001, 17:331–337
21. Brabin BJ: An analysis of malaria in pregnancy in Africa. Bull WHO 1983, 61:1005–1016
22. Bray RS, Sinden RE: The sequestration of Plasmodium falciparum infected erythrocytes in the placenta. Trans R Soc Trop Med Hyg 1979, 73:716–719

23. Menendez C, Ordi J, Ismail MR, Ventura PJ, Aponte JJ, Kahigwa E, Font F, Alonso PL: The impact of placental malaria on gestational age and birth weight. J Infect 2000, 181:1740–1745
24. McGregor IA, Wilson ME, Billewicz WZ: Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birth weight and placental weight. Trans R Soc Trop Med Hyg 1983, 77:232–244
25. Beeson JG, Amin N, Kanjala M, Rogerson SJ: Selective accumulation of mature asexual stages of Plasmodium falciparum-infected erythrocytes in the placenta. Infect Immun 2002, 70:5412–5415
26. Duffy PE, Fried M: Malaria In Pregnancy: Deadly Parasite, Susceptible Host. Edited by PE Duffy, M Fried. New York, Taylor and Francis Press, 2001, pp 1–245
27. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE: Maternal antibodies block malaria. Nature 1998, 395:851–852
28. Gysin J, Pouvelle B, Fievet N, Scherf A, Lepolard C: Ex vivo desequestration of P. falciparum-infected erythrocytes from human placenta by CSA. Infect Immun 1999, 67:6596–6602
29. Maubert B, Fievet N, Tami G, Cot M, Boudin C, Deloron P: Development of antibodies against chondroitin sulfate A-adherent Plasmodium falciparum in pregnant women. Infect Immun 1999, 67:5367–5371
30. Ricke CH, Staalsoe T, Koram K, Akanmori BD, Riley EM, Theander TG, Hviid L: Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on P. falciparum-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. J Immunol 2000, 165:3309–3316
31. Staalsoe T, Megnekou R, Fievet N, Ricke CH, Zornig HD, Leke R, Taylor DW, Deloron P, Hviid L: Acquisition and decay of antibodies to pregnancy-associated variant antigens to the surface of Plasmodium falciparum-infected erythrocytes that protect against placental parasitemia. J Infect Dis 2001, 184:618–626
32. O'Neil-Dunne I, Achur RN, Agbor-Enoh ST, Valiyaveettil M, Naik RS, Ockenhouse CF, Zhou A, Megnekou R, Leke R, Taylor DW, Gowda DC: Gravidity-dependent production of antibodies that inhibit binding of Plasmodium falciparum-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy. Infect Immun 2001, 69:7487–7492
33. Fievet N, Tami G, Maubert B, Moussa M, Shaw IK, Cot M, Holder AA, Chaouat G, Deloron P: Cellular immune response to Plasmodium falciparum after pregnancy is related to previous infection and parity. Malaria J 2002, 1:16–23
34. Fried M, Duffy PE: Adherence of Plasmodium falciparum to chondroitin sulphate A in the human placenta. Science 1996, 272:1502–1504
35. Maubert B, Fievet N, Tami G, Boudin C, Deloron P: Cytoadherence of Plasmodium falciparum-infected erythrocytes in the placenta. Parasite Immunol 2002, 22:191–199
36. Pouvelle B, Fusai T, Lepolard C, Gysin J: Biological and biochemical characteristics of cytoadhesion of Plasmodium falciparum-infected erythrocytes to chondroitin-4-sulfate. Infect Immun 1998, 66:4950–4956
37. Rogerson SJ, Brown GV: Chondroitin sulphate A as an adherence receptor for Plasmodium falciparum-infected erythrocytes. Parasitol Today 1997, 13:70–75
38. Alkhalil A, Achur RN, Valiyaveettil M, Ockenhouse CF, Gowda DC: Structural requirements for the adherence of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate proteoglycans of human placenta. J Biol Chem 2000, 275:40357–40364
39. Chai W, Beeson JG, Lawson AM: The structural motif in chondroitin sulfate for adhesion of Plasmodium falciparum-infected erythrocytes comprises disaccharide units of 4-O-sulfated and non-sulfated N-acetylgalactosamine linked to glucuronic acid. J Biol Chem 2002, 277:22438–22446
40. Walter PR, Garin Y, Blot P: Placental pathologic changes in malaria: a histologic and ultrastructual study. Am J Pathol 1982, 109:330–342
41. Yamada M, Stekete R, Abramowsky C, Kida M, Wirima J, Heymann D, Rabbege J, Breman J, Aikawa M: Plasmodium falciparum associated placental pathology: a light and electron microscopic and immunohistologic study. Am J Trop Med Hyg 1989, 41:161–168
42. Duffy PE, Fried M: Turncoat antibodies. Science 2001, 293:2009–2010
43. Miller LH, Smith JD: Motherhood and malaria. Nat Med 1998, 4:1244–1245
44. Achur RN, Valiyaveettill M, Alkhalil A, Ockenhouse CF, Gowda DC: Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycan of the intervillous spaces that mediates the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. J Biol Chem 2000, 275:40344–40356
45. Oike Y, Kimata K, Shinomura T, Nakazawa K, Suzuki S: Structural analysis of chick-embryo cartilage proteoglycan by selective degradation with chondroitin lyases (chondroitinases) and endo-beta-D-galactosidase (keratinases). Biochem J 1980, 191:193–207
46. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
47. Krueger Jr RC, Schwartz NB: An improved method of sequential Alcian Blue and ammoniacal silver staining of chondroitin sulfate proteoglycan in polyacrylamide gel. Anal Biochem 1987, 167:295–300
48. Buckley MS, Washington S, Laurent C, Erickson DR, Bhavanandan VP: Characterization and immunohistochemical localization of the glycoconjugates of the rabbit bladder mucosa. Arch Biochem Biophys 1996, 330:163–173
49. Laurent C, Hellstrom S, Laurent E, Wells AF, Bergh A: Localization and quantity of hyaluronan in urogenital organs of male and female rats. Cell Tissue Res 1995, 279:241–248
50. Couchman JR, Caterson B, Christner JE, Baker JR: Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. Nature 1984, 307:650–652
51. Sorrell JM, Carrino DA, Baber MA, Asselineau D, Caplan AI: A monoclonal antibody which recognizes a glycosaminoglycan epitope in both dermatan sulfate and chondroitin sulfate proteoglycans of human skin. Histochem J 1999, 31:549–558
52. Fisher LW: Decorin. Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins. Edited by T Kreis, R Vale. New York, Sambrook and Tooze Publication, Oxford University Press, 1999, pp 408–411
53. Maruyama I, Bell CE, Majerus PW: Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. J Cell Biol 1985, 101:363–371
54. Lin JH, McLean K, Morser J, Young TA, Wydro RM, Andrews WH, Light DR: Modulation of glycosaminoglycan addition in naturally expressed and recombinant human thrombomodulin. J Biol Chem 1994, 269:25021–25030
55. Achur RN, Valiyaveettil M, Gowda DC: The low sulfated chondroitin sulfate proteoglycans of human placenta have sulfate group-clustered domains that can efficiently bind Plasmodium falciparum-infected erythrocytes. J Biol Chem 2003, 278:11705–11713